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(54) **MULTIPLEX FLOW IMMUNOASSAYS WITH MAGNETIC PARTICLES AS SOLID PHASE**

MULTIPLEX-ZUFLUSS-IMMUNOTEST MIT MAGNETISCHEN TEILCHEN ALS FESTPHASE

DOSAGES IMMUNOLOGIQUES MULTIPLEX DE FLUX, DANS LESQUELS DES PARTICULES MAGNETIQUES FONT OFFICE DE PHASE SOLIDE

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- **CHEMICAL ABSTRACTS, vol. 117, no. 11, 14 September 1992 Columbus, Ohio, US; abstract no. 110242, J. E. RESELAND ET AL.: "Use of flow cytometry for detection and quantitation of Clostridium perfringens type A enterotoxin-positive spores." page 733; column 1; XP002095326 & FOOD SAF. QUAL. ASSUR.: APPL. IMMUNOASSAY SYST., PROC., 1ST.,1991, pages 315-323,**

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Description

[0001] This invention resides in the field of clinical assays indicative of biological conditions, and is of interest in the technology of binding assays for analytes in biological fluids for purposes of diagnosis, monitoring, or other clinical functions.

[0002] Since the initial disclosure of radioimmunoassays in 1961, a wide variety of *in vitro* assays using affinity-type binding have been developed. Variations include the type of binding (for example, specific *vs.* non-specific, and immunological *vs.* non-immunoiogical), the type of detection (including the use of labels such as enzyme labels, radioactive labels, fluorescent labels, and chemiluminescent labels), methods of detecting whether or not binding has occurred (including methods in which bound species are separated from unbound species and methods that do not include such separation), and various other aspects of the assay procedure. The technology is currently used for the detection and quantitation of countless species, and serves as an analytical tool in the detection and monitoring of many physiological conditions and functions and the diagnosis and treatment of many diseases.

[0003] Improvements in the efficiency and reproducibility of these assays have been made by various developments including improved labels, methods of detection, automation, and systems for multiplex analyses. Each procedure however requires a sequence of steps, and any means of shortening the sequence, increasing the number of analyses that can be performed within a given period of time, or improving the reproducibility and versatility of the assay will benefit the purpose of the assay.

[0004] Many binding assays are heterogeneous assays, which rely in part on the transfer of analyte from a liquid sample to a solid phase by the binding of the analyte during the assay to the surface of the solid phase. At some stage of the assay, whose sequence varies depending on the assay protocol, the solid phase and the liquid phase are separated and the determination leading to detection and/or quantitation of the analyte is performed on one of the two separated phases. One type of solid phase that has been used are magnetic particles, which offer the combined advantages of a high surface area and the ability to be temporarily immobilized at the wall of the assay receptacle by imposition of a magnetic field while the liquid phase is aspirated, the solid phase is washed, or both. Descriptions of such particles and their use are found in Forrest *et al.*, United States Patent No. 4,141,687 (Technicon Instruments Corporation, February 27, 1979); Ithakissios, United States Patent No. 4,115,534 (Minnesota Mining and Manufacturing Company, September 19, 1978); Vlieger, A.M., *et al., Analytical Biochemistry* **205**:1-7 (1992); Dudley, *Journal of Clinical Immunoassay* **14**:77-82 (1991); and Smart, *Journal of Clinical Immunoassay* **15**:246-251(1992). Such magnetic methods should not, however, be confused with the use of magnetic particles in chromatographic techniques, such as that referred to as highgradient magnetic differentiation in WO 90/07380.

[0005] Of further possible relevance to this invention is the state of the art relating to the use of flow cytometry for the detection and analysis of particles and species bound to the particles. Flow cytometry has been disclosed for use in the detection and separation of antigens and antibodies by Coulter Electronics Inc., United Kingdom Patent No. 1,561,042 (published February 13, 1980); and for quantitation of PCR (Polymerase Chain Reaction) products by Vlieger, A.M., *et al.*, *Analytical Biochemistry* **205**:1-7 (1992). Flow cytometry has been limited in the analysis of biological samples. The sensitivity of those assay formats that do not require separation of free from bound species (i.e., sandwich and competitive assays) is adversely affected by the increased background signal noise caused by the unbound label. Antigen-capture antibody assays require the removal of non-specific immunoglobulin before the addition of class-specific labeled anti-Ig. Samples containing particulates (such as stool samples, for example) require the removal of this debris which would otherwise interfere with the flow cytometric measurement. Traditional separation techniques, such as filtration or centrifugation would be successful in removing unbound label or non-specific Ig but would fail to remove interfering particulates from the patient sample. In addition, these traditional separation techniques are difficult and/or costly to automate. The use of magnetic particles and magnetism is a well known method and has been shown to be both efficient and cost-effective in automated diagnostic systems.

[0006] This invention resides in an assay that combines multiplexing of heterogeneous binding assays of a single fluid sample by flow cytometry with the use of solid magnetic particles as the solid phase to facilitate the separation of solid and liquid phases. The magnetic particles have sizes that are microscopic (and hence termed "microparticles") and that vary over a size range that is an aggregate of two or more smaller size ranges, referred to herein as "subranges." The subranges are substantially discrete (nonoverlapping), with the mean particle sizes of adjacent subranges sufficiently far apart to permit differentiation of each subrange from the others by flow cytometry. An assay reagent is bonded to each particle, with substantially all particles within each subrange bearing the same assay reagent and with different assay reagents from one subrange to the next. The subranges are thus distinguishable not only by size for purposes of flow cytometry but also by the assay reagents bonded to the particles such that all particles in each subrange take part in a distinct binding assay, and do so in a selective manner relative to the assay reagents bonded to particles in other subranges.

[0007] The magnetic character of the particles permits the automated separation of solid phase from liquid phase at a point in the sequence of the assay prior to the flow cytometry stage. The separation can serve any of a variety of

purposes, including the removal of sample debris from the assay components, the removal of sample components that would otherwise contribute significantly to the background noise at the detection stage, the removal of competing binding members that are not the subject of any of the assays but would otherwise interfere with the results, and the removal of bound from unbound species such as labels, analytes, analyte binding members, and label-binding member conjugates. The particular function in any given assay or combination of assays will depend on the nature of the assay and the assay protocol.

[0008] These and other features and advantages of the invention will be more readily understood by the description that follows.

[0009] The term "magnetically responsive material" is used herein to denote a material that responds to a magnetic field. Magnetically responsive materials of interest in this invention include paramagnetic materials, ferromagnetic materials, ferrimagnetic materials, and metamagnetic materials. Paramagnetic materials are preferred. Examples are iron, nickel, and cobalt, as well as metal oxides such as Fe_3O_4, $BaFe_{12}O_{19}$, CoO, NiO, Mn_2O_3, Cr_2O_3, and CoMnP. Rather than constituting the entire microparticle, the magnetically responsive material is preferably only one component of the microparticle whose remainder consists of a polymeric material to which the magnetically responsive material is affixed and which is chemically derivatized to permit attachment of an assay reagent.

[0010] The quantity of magnetically responsive material in the microparticle is not critical and can vary over a wide range, although the quantity can affect the density of the microparticle, which in conjunction with the particle size can affect the ease of maintaining the microparticle in suspension for purposes of achieving maximal contact between the liquid and solid phase and for facilitating flow cytometry. Furthermore, an excessive quantity of magnetically responsive material in the microparticles will produce autofluorescence at a level high enough to interfere with the assay results. It is therefore preferred that the concentration of magnetically responsive material be low enough to minimize any autofluorescence emanating from the material. With these considerations in mind, the magnetically responsive material in a microparticle in accordance with this invention preferably ranges from about 1% to about 75% by weight of the particle as a whole. A more preferred weight percent range is from about 2% to about 50%, a still more preferred weight percent range is from about 3% to about 25%, and an even more preferred weight percent range is from about 5% to about 15%. The magnetically responsive material can be dispersed throughout the polymer, applied as a coating on the polymer surface or as one of two or more coatings on the surface, or incorporated or affixed in any other manner that secures the material to the polymer.

[0011] The polymeric matrix that forms the remainder of the microparticle can be any material that can be formed into a microparticle, that, other than the assay reagent that is affixed to the microparticle, is inert to the components of the biological sample and to the other assay reagents, that has minimal autofluorescence, that is solid and insoluble in the sample and in any other solvents or carriers used in the assay, and that is capable of affixing an assay reagent to the microparticle. Examples of suitable polymers are polyesters, polyethers, polyolefins, polyalkylene oxides, polyamides, polyurethanes, polysaccharides, celluloses, and polyisoprenes. Crosslinking is useful in many polymers for imparting structural integrity and rigidity to the microparticle.

[0012] Functional groups for attachment of the assay reagent can be incorporated into the polymer structure by conventional means, including the use of monomers that contain the functional groups, either as the sole monomer or as a co-monomer. Examples of suitable functional groups are amine groups ($-NH_2$), ammonium groups ($-NH_3^+$ or $-NR_3^+$), hydroxyl groups (—OH), carboxylic acid groups (—COOH), and isocyanate groups (—NCO). A useful monomer for introducing carboxylic acid groups into polyolefins, for example, is acrylic acid or methacrylic acid.

[0013] Attachment of the assay reagent to the microparticle surface can be achieved by electrostatic attraction, specific affinity interaction, hydrophobic interaction, or covalent bonding. Covalent bonding is preferred. Linking groups can be used as a means of increasing the density of reactive groups on the microparticle surface and decreasing steric hindrance to increase the range and sensitivity of the assay, or as a means of adding specific types of reactive groups to the microparticle surface to broaden the range of types of assay reagents that can be affixed to the microparticle surface. Examples of suitable useful linking groups are polylysine, polyaspartic acid, polyglutamic acid and polyarginine.

[0014] In some cases, particles possess high autofluorescence and as such are unsuitable for use in a flow cytometric immunoassay. Particles created by standard emulsion polymerization techniques from a wide variety of starting monomers in general exhibit low autofluorescence. Particles whose surfaces have been specifically modified to increase porosity and therefore surface area (such particles are referred to in the literature as "macroporous" particles) exhibit high autofluorescence. Autofluorescence in such particles increases with increasing size and increasing percentage of divinylbenzene monomer.

[0015] With these considerations in mind, the size range of the microparticles can vary and particular size ranges are not critical to the invention. In most cases, the aggregated size range of the microparticles lies within the range of from about 0.3 micrometers to about 100 micrometers in particle diameter, and preferably within the range of from about 0.5 micrometers to about 40 micrometers. The subranges are two or more in number, and in most cases will be from two to twenty, each selectively active in a single assay and inert relative to the other assays simultaneously being

performed or detected.

[0016] The widths of the subranges and the spacing between mean diameters of adjacent subranges are selected to permit differentiation of the subranges by flow cytometry, and will be readily apparent to those skilled in the use of and in the instrumentation for flow cytometry. In this specification, the term "mean diameter" refers to a number average diameter. In most cases, a preferred subrange width is about ±5% CV or less of the mean diameter, where CV is the coefficient of variation and is defined as the standard deviation of the particle diameter divided by the mean particle diameter times 100 percent. The minimum spacing between mean diameters among the various subranges can vary depending on the microparticle size distribution, the ease of segregating microparticles by size for purposes of attaching different assay reagents, and the type and sensitivity of the flow cytometry equipment. In most cases, best results will be achieved when the mean diameters of different subranges are spaced apart by at least about 6% of the mean diameter of one of the subranges, preferably at least about 8% of the mean diameter of one of the subranges and most preferably at least about 10% of the mean diameter of one of the subranges. Another preferred subrange width relation is that in which the standard deviation of the particle diameters within each subrange is less than one third of the separation of the mean diameters of adjacent subranges.

[0017] The type of assay reagent attached to the microparticle surface for any single subrange of microparticles will vary depending on both the analyte and the type of assay. The assay reagent can be a binding agent with specific affinity for the analyte, or a binding agent with affinity for a narrow range of species that includes the analyte but excludes other analytes whose assays are performed by contact with other microparticle subranges, or any binding species in general that will selectively engage in the assay for a single analyte to the exclusion of the others. Examples of assay reagents are antibodies, antigens or haptens, and other types of proteins with binding specificity such as avidin and biotin.

[0018] Another type of assay reagent that can be attached to the microparticle surface for any single subrange of microparticles is the analyte itself. In the analysis, the attached analyte will compete with a narrow range of species in the sample that also includes analyte. Examples of these assay reagents are antibodies, antigens and haptens.

[0019] The signal due to a specific analyte may be differentiated, singly or in combination, by the following parameters: the size of the particle (as reflected in the low and high angle scatter measurements), the composition of the particle (reflected by changes in the high angle scatter), or the fluorescence wavelength of the label (in which the different wavelengths are separated by dichroic filters and detected by the fluorescence photomultiplier tubes).

[0020] The assay performed at the surfaces of microparticles within a single subrange can be any type of heterogeneous assay that yields a result differentiating a certain analyte from others in the sample.

[0021] Competitive assays, for example, can be performed by using magnetically responsive microparticles to which are bound molecules of a binding protein (such as an antibody) specific for the analyte. During the assay, the sample and a quantity of labeled analyte, either simultaneously or sequentially, are mixed with the microparticles. By using a limited number of binding sites on the microparticles, the assay causes competition between the labeled analyte and the analyte in the sample for the available binding sites. After a suitable incubation period, the mixture of liquid and solid is placed under the influence of a magnetic field, causing the microparticles to adhere to the walls of the reaction vessel, and the liquid phase is removed. The microparticles, still adhering to the vessel wall, are then washed to remove any remaining unbound analyte and label, and resuspended in a carrier liquid for introduction into a flow cytometer where the microparticles are classified by size and the label detected. An example of an analyte that is readily detected in this manner is vitamin B_{12}. A useful particle-bound assay reagent for this analyte is B_{12} intrinsic factor, and a competing label-bound analyte is B_{12} covalently linked to phycoerythrin.

[0022] Immunometric or sandwich assays, as another example, are performed by using magnetically responsive microparticles to which are bound antibodies to the analyte. In this case, the bound antibodies are present in excess relative to the suspected quantity range of the analyte so that all of the analyte binds. The microparticles are placed in contact with the sample, and simultaneously or sequentially, a second antibody to same analyte is added, again in excess relative to the analyte, the first and second antibodies binding different epitopes on the analyte in a non-interfering manner, and the second antibody being conjugated to a detectable label. After a suitable incubation period, the liquid mixture with microparticles suspended therein is placed under the influence of a magnetic field, causing the microparticles to adhere to the walls of the reaction vessel, and the liquid phase is removed. The microparticles, still adhering to the vessel wall, are then washed to remove excess amounts of the second, labeled antibody that have not become bound to the immobilized analyte, and the microparticles are then resuspended in a carrier liquid for introduction into a flow cytometer where they are sorted by size and the label detected. An example of an analyte that is readily detected in this manner is thyroid stimulating hormone (TSH). The label on the second antibody can again be phycoerythrin.

[0023] This invention can also be applied to assays that do not separate bound label from unbound label but nevertheless require separation of the solid from the liquid phase at some point in the assay. Examples are assays for identifying antibodies to infectious diseases. The analyte antibodies bind to particle-bound antigens in the assay, and are followed by labeled binding members that bind to the analyte antibodies that have thus become attached to the

solid phase. The antigens originally bonded to the solid phase are done so in a high density so that any label that subsequently attaches to the solid phase is sufficiently concentrated at the solid phase surface to be distinguishable from unbound label in the solution. While this eliminates the need for separating bound from unbound label. it still requires the removal of other species from the sample that would compete with the labeled secondary antibodies that bind to the analyte antibodies which are bound to the particle-bound antigen. The magnetic particles serve this purpose as in the assays described in the preceding paragraphs. and this is performed before the label is added.

[0024] A different type of serological assay for antibodies are a further example, performed by using magnetically responsive microparticles to which are bound antibodies to the antibody analyte. The microparticles are placed in contact with the sample. After a suitable incubation period, the liquid mixture with suspended microparticles is placed under a magnetic field to adhere the microparticles to the reaction vessel walls. and the liquid phase is removed. Labelled antigen is then added to the vessel containing the microparticles, the antigen being the one that the analyte antibodies are directed towards and that is conjugated to a detectable label or is attached through other binding pairs. After a suitable incubation period. this new liquid mixture is introduced into a flow cytometer where the microparticles are classified by size and the label detected. An example of an analyte susceptible to this type of assay is human anti-Rubella IgG. The particle-bound reagent is Rubella antigen and the labeled secondary antibody is anti-human IgG antibody covalently linked to phycoerythrin.

[0025] The multiple assays that can be performed on a single fluid sample in accordance with this invention can be all of the same type (*i.e.*, all competitive, all immunometric, all serological, etc.) or a combination of different types. Examples of combinations of assays that can be performed by this method are:

(1) Assays for thyroid stimulating hormones and either free T_4 or total T_4;
(2) Assays for vitamin B_{12} and folate; and
(3) TORCH assays. detecting serum IgG and serum IgM responses to *Toxoplasma gondii*, Rubella virus, Cytomegalovirus, and Herpes Simplex Virus Types 1 and 2.

[0026] Methods of and instrumentation for flow cytometry are known in the art, and those that are known can be used in the practice of the present invention. Flow cytometry in general resides in the passage of a suspension of the microparticles as a stream past electro-optical sensors, in such a manner that only one particle at a time passes the sensors. As each particle passes the sensors, the particle produces a signal due to light scattering, the amplitude of the signal varying with the particle size. The signals are classified by the instrumentation according to their amplitudes, the particles thereby differentiated according to size. The presence and amount of label on each particle is also detected by its fluorescence and correlated with the particle size subrange so that individual assay results are achieved for each particle size subrange. Descriptions of instrumentation and methods for flow cytometry are found in the literature. Examples are McHugh, "Flow Microsphere Immunoassay for the Quantitative and Simultaneous Detection of Multiple Soluble Analytes." *Methods in Cell Biology* **42**, Part B (Academic Press, 1994); McHugh *et al.*, "Microsphere-Based Fluorescence Immunoassays using Flow Cytometry Instrumentation," *Clinical Flow Cytometry,* Bauer, K.D., *et al.*, eds. (Baltimore, Maryland. USA: Williams and Williams, 1993), pp. 535-544; Lindmo *et al.*, "Immunometric Assay Using Mixtures of Two Particle Types of Different Affinity," *J. Immunol. Meth.* **126**: 183-189 (1990); McHugh, "Flow Cytometry and the Application of Microsphere-Based Fluorescence Immunoassays." *Immunochemica* **5**: 116 (1991); Horan *et al.*, "Fluid Phase Particle Fluorescence Analysis: Rheumatoid Factor Specificity Evaluated by Laser Flow Cytophotometry," *Immunoassays in the Clinical Laboratory*, 185-189 (Liss 1979); Wilson *et al.*, "A New Microsphere-Based Immunofluorescence Assay Using Flow Cytometry, *J. Immunol. Meth.* **107**: 225-230 (1988); Fulwyler *et al.*, "Flow Microsphere Immunoassay for the Quantitative and Simultaneous Detection of Multiple Soluble Analytes," *Meth. Cell Biol.* **33**: 613-629 (1990); Coulter Electronics Inc., United Kingdom Patent No. 1.561.042 (published February 13, 1980); and Steinkamp *et al.*, *Review of Scientific Instruments* **44**(9): 1301-1310 (1973).

[0027] Similarly. methods of and instrumentation for applying and removing a magnetic field as part of an automated assay are Known to those skilled in the an and reported in the literature. Examples of literature reports are the Forrest *et al.* patent. the Ithakissios patent. the Vlieger *et al.* paper. the Dudley paper and the Smart paper, all referenced above.

[0028] This invention is applicable to the analysis of biological fluids, notably physiological fluids such as whole blood, serum, urine, spinal fluid. saliva. and stool samples.

[0029] The following examples are offered strictly for purposes of illustration.

EXAMPLE 1

[0030] This example illustrates the attachment of viral antigen (rubella (RUB), cytomegalovirus (CMV) and herpes simplex virus 2 (HSV2)) to magnetic beads.

[0031] Three types of magnetic beads were used:

SPHERO™ Carboxyl Magnetic particles, from Spherotech, Inc., Libenyville, Illinois, USA -- poly(styrene/acrylic acid particles), 4.35 micrometers (μm) in diameter, density 1.17 g/cc, containing 12% magnetite (by weight)
SPHERO™ Carboxyl Magnetic particles, from Spherotech, Inc., Libertyville, Illinois, USA -- poly(styrene/acrylic acid particles), 3.18 μm in diameter, density 1.17 g/cc, containing 12% magnetite (by weight)
SINTEF Applied Chemistry, Trondheim, Norway -- poly(styreneldivinylbenzene) particles, 10 μm in diameter, density 1.23 g/cc, containing 17.9% magnetite maghemite (by weight)

[0032] Table I lists the amounts of each of the materials used in this preparation:

TABLE I

Amounts Used					
Bead	Viral Antigen	Amount of Beads	Wt. of Viral Antigen	Volume of Viral Antigen	Volume of Phosphate Buffer (100 mM)
4.35μm	CMV	10 mg	225.8 μg	322.6 μL	677.4 μL
3.18μm	HSV2	5 mg	163.0 μg	815.0 μL	185.0 μL
10 μm	RUB	5 mg	5.2 μg	104.0 μL	896.0 μL

[0033] The beads in each case were placed in test tubes and washed multiple times with 100 mM phosphate buffer, pH 6.8. The washed beads were then suspended in the volume of phosphate buffer listed in Table I, and respective antigen solution was added (CMV antigen from Chemicon International Incorporated, Temecula, California, USA; HSV2 antigen from Ross Southern Labs, Salt Lake City, Utah, USA; and RUB antigen from Viral Antigens, Memphis, Tennessee, USA) in the amount listed in the Table. The test tubes were then rotated in end-over-end fashion overnight at room temperature. The tubes were then placed on a magnetic separator and the supernatant was drawn off and discarded. The resulting beads were washed with a wash buffer consisting of 50 mM phosphate buffer, pH 7.4, 0.01% Tween 20, 1% bovine serum albumin, 0.1% sodium azide, 150 mM sodium chloride, then again subjected to magnetic separation, and suspended in a storage buffer consisting of 50 mM phosphate buffer, pH 7.4, 5% glycerol, 1 % bovine serum albumin, 0.1% sodium azide, 150 mM sodium chloride.

EXAMPLE 2

[0034] This example illustrates the use of the CMV-coated magnetic beads of Example 1 in a flow cytometric immunoassay.

Procedure:

[0035]

1. 100 μL of Bio-Rad CMV IgG Immunoassay positive and negative controls (Bio-Rad Laboratories, Inc., Hercules, California, USA, diluted 1:10 in wash buffer) were added to 12 x 75 mm polypropylene test tubes.
2. To each tube was added 100 μL of the CMV antigen-coated particles (described in Example 1) diluted 1:1000 in wash buffer.
3. The tubes were vortexed at ambient temperature for 30 minutes.
4. After vortexing, 800 μL of wash buffer was added to each tube.
5. The tubes were placed in a magnetic separator for 3 minutes and the liquid phase removed.
6. Steps 4 and 5 are repeated but with 1000 μL of wash buffer.
7. 200 μL of a 1:100 dilution of anti human IgG-phycoerythrin conjugate (Chemicon International Inc., Temecula, California, USA) is added.
8. The tubes were vortexed at ambient temperature for 30 minutes.
9. After this time, the samples are injected into the flow cytometer (Bryte HS, Bio-Rad Laboratories, Inc., Hercules, California, USA) equipped with a Xenon arc lamp.

Results:

[0036] Positive and negative CMV controls exhibited fluorescent peaks corresponding to 898 and 60 relative linear fluorescence units, respectively. As expected, the positive control gave significantly elevated signal relative to that of

the negative control.

EXAMPLE 3

[0037] This example illustrates the use of the CMV, HSV2 and RUB-coated magnetic particles of Example 1 in a simultaneous multi-analyte flow cytometric immunoassay.

Procedure:

[0038]

1. 100 µL of patient samples (diluted 1:10 in wash buffer), of known CMV, HSV2 and RUB antibody status, were added to 12 x 75 mm polypropylene test tubes.
2. To each tube was added 100 µL of a mixture of CMV, HSV2 and RUB antigen-coated particles (described in Example 1) diluted in wash buffer.
3. The tubes were vortexed at ambient temperature for 15 minutes.
4. After vortexing, 800 µL of wash buffer was added to each tube.
5. The tubes were placed in a magnetic separator for 5 minutes and the liquid phase removed.
6. Steps 4 and 5 are repeated but with 1000 µL of wash buffer.
7. 200 µL of a 1:300 dilution of anti-human IgG-phycoerythrin conjugate (Chemicon International Inc., Temecula, California, USA) is added.
8. The tubes were vortexed at ambient temperature for 15 minutes.
9. After this time, the samples are injected into the flow cytometer (Bryte HS, Bio-Rad Laboratories, Inc., Hercules, California, USA) equipped with a Xenon arc lamp.

Results:

[0039] The results are summarized in Table II below:

TABLE II

Test Results						
Sample	Antibody Status			Relative Linear Fluorescence Units		
	CMV	HSV2	RUB	CMV	HSV2	RUB
CN6	+	-	+	14	7	155
Cn8	+	-	+	16	6	181
CN12	-	-	+	5	7	240
CN15	-	-	+	5	6	329
23	-	+	-	5	45	43

[0040] The data in Table II show that positive samples have substantially increased fluorescence relative to the negative samples.

EXAMPLE 4

[0041] This example illustrates the covalent attachment of rubella (RUB) antigen to magnetic beads.

[0042] The magnetic particles were SPHERO™ Carboxyl Magnetic particles, from Spherotech, Inc., Libertyville, Illinois, USA -- poly(styrene/alkylenic acid particles), 25 mg/mL, 7.1 micrometers in diameter, density 1.165 g/cc, containing 12% magnetite (by weight).

[0043] 1.13 mL of beads were placed in a test tube and washed multiple times with 50 mM 2-(N-morpholino) ethanesulfonic acid (MES) buffer, pH 5.5. The washed beads were then suspended in 1.25 mL of 2 mg/mL polylysine (MW 18,000) in 50 mM MES buffer, pH 5.5. To the resulting solution was added 125 µL of 20 mg/mL 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide (EDC) in water. The solution was placed on an end-over-end rotator at ambient temperature for 18 hours. After this time the solution was separated from the particles and discarded. The particles were then washed multiple times with 0.2M borate buffer, pH 8.5. The particles were resuspended in 2.5 mL of borate buffer, pH 8.5. To this solution was added 50 mg of succinic anhydride. The solution was then placed on an end-over-end

rotator at ambient temperature for 4 hours. After this time the solution was separated from the particles and discarded. The particles were then washed multiple times with 50 mM MES. pH 5.5. The particles were washed twice with 0.1M carbonate buffer. pH 9.6 and then 3 times with 20mM phosphate buffer, pH 4.5. The particles were finally suspended in 1 mL of 20 mM phosphate buffer, pH 4.5. To this solution was added 1 mL of 20 mg/mL EDC in 20 mM phosphate buffer, pH 4.5. The solution was then placed on an end-over-end rotator at ambient temperature for 4 hours. After this time the solution was separated from the particles and discarded. The particles were washed 3 times with 20mM phosphate buffer, pH 4.5. Afterwards the beads were suspended in 2 mL of 0.2M borate buffer, pH 8.5. To this was added 0.5 mL of 0.2 mg/mL of rubella antigen from Viral Antigens Incorporated. Memphis. Tennessee. USA in 0.2M borate buffer. pH 8.5. 2 mg/mL 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate (CHAPS). 0.1% sodium azide. The test tube were then rotated in end-over-end fashion overnight at room temperature. The next day, 100 µL of 0.25M hydroxylamine in 0.2M borate buffer, pH 8.5 was introduced. The solution was then placed on an end-over-end rotator at ambient temperature for 1 hour. After this time the solution was separated from the particles and discarded. The particles were washed 3 times with wash buffer (see Example 1). The resulting beads were taken up in 2.5 mL of wash buffer and placed on an end-over-end rotator at ambient temperature for 1 hour. After this time the solution was separated from the particles and discarded. The particles were washed 3 times with storage buffer (see Example 1). Finally the particles were suspended in 1 mL of storage buffer and placed at 4°C.

EXAMPLE 5

[0044] This example illustrates the use of magnetic particles with covalently attached rubella antigen of Example 4 in a quantitative flow cytometric immunoassay.

Procedure:

[0045]

1. 100 µL of Bio-Rad RUB IgG Immunoassay standards. high positive. low positive and negative controls (Bio-Rad Laboratories, Inc., Hercules, CA, diluted 1:30 in wash buffer), were added to 12x75mm polypropylene test tubes.
2. To each tube was added 100 µL of the RUB antigen-coated particles (described above) diluted 1:200 in wash buffer.
3. The tubes were vortexed at ambient temperature for 15 minutes.
4. After vortexing, 750 µL of wash buffer was added to each tube.
5. The tubes were placed in a magnetic separator for 1 minute and the liquid phase removed.
6. Steps 4 and 5 are repeated two more times but with 1000 µL of wash buffer.
7. 200 µL of a 1:300 dilution of anti human IgG-phycoerythrin conjugate (Chemicon International Inc., Temecula, California, USA) is added.
8. The tubes were vortexed at ambient temperature for 15 minutes.
9. The samples are then injected into the flow cytometer (Bryte HS, Bio-Rad Laboratories, Inc., Hercules, California, USA) equipped with a Xenon/Mercury arc lamp.

Results:

[0046] Table III contains data generated by following the above protocol. The standards were fitted to a 4-parameter logistic equation. The concentrations of all samples were calculated from this curve. The values for the controls are similar to the values assigned by the Bio-Rad ELISA technique.

TABLE III

Test Results			
Sample	Relative Linear Fluorescence Units	Observed [RUB] (IU/mL)	Reported [RUB] (IU/mL)
Standard 0	21	0	0
Standard 1	101	6	8
Standard 2	225	30	30
Standard 3	464	115	96
Standard 4	652	216	240
Standard 5	1171	623	614

TABLE III (continued)

Test Results			
Sample	Relative Linear Fluorescence Units	Observed [RUB] (IU/mL)	Reported [RUB] (IU/mL)
High Positive	580	174	135
Low Positive	175	18	14
Negative	33	0.3	0.5

Claims

1. A method for individually detecting a plurality of analytes in a single fluid biological sample by assays that include the binding of species in single fluid biological sample to a solid phase that is in contact with a liquid medium in which said solid phase is insoluble and the separation of said solid phase from said liquid medium, said method comprising:

   using as said solid phase a plurality of microparticles of magnetically responsive material each with an assay reagent coupled thereto that is selectively active in an assay for one of said plurality of analytes, said microparticles varying in size over a range that is an aggregate of a plurality of subranges, each subrange distinguishable from other subranges of said aggregate by flow cytometry and by the assay reagent coupled to the microparticles of said subrange;
   magnetically separating microparticles in all of said subranges from said liquid medium in a single step, and defining said liquid medium as a first liquid medium, suspending said microparticles separated therefrom in a second liquid medium, analyzing said microparticles in said second liquid medium in a single stop by flow cytometry in accordance with said plurality of assays, thereby achieving individual detection of said plurality of analytes in said single fluid biological sample.

2. A method in accordance with claim **1** in which one of said assay reagents coupled to said solid phase is a binding protein specific for one of said analytes, said method includes adding to said first liquid medium a detectable label that binds to said solid phase, and said magnetic separation comprises separating detectable label that is bound to said solid phase from unbound detectable label that is suspended in said first liquid medium.

3. A method in accordance with claim **2** in which said detectable label is added to said first liquid medium as a conjugate with an additional quantity of said one analyte, causing said conjugate and said one analyte in said sample to compete for said binding protein in a competitive assay.

4. A method in accordance with claim **2** in which said detectable label is added to said first liquid medium as a conjugate with an additional quantity of said one analyte after a first incubation period, causing said conjugate to react with those sites of said binding protein not occupied by said one analyte in said sample in a sequential assay.

5. A method in accordance with claim **2** in which said binding protein is defined as a first binding protein, and said detectable label is added to said first liquid medium as a conjugate with a second binding protein that is also specific for said one analyte, causing said one analyte to bind to both said first binding protein and said conjugate in a sandwich assay.

6. A method in accordance with claim **5** in which said one analyte is an antigen, said first binding protein is a first antibody to said antigen, and said second binding protein is a second antibody to said antigen.

7. A method in accordance with claim **5** in which said one analyte is a first antibody, said first binding protein is an antigen, and said second binding protein is a second antibody having binding affinity for said first antibody.

8. A method in accordance with claim **1** in which one of said assay reagents is a binding protein bound to said solid phase and specific for one of said analytes and for other components in said first liquid medium, and said magnetic separation comprises separating said microparticles with said one analyte bound thereto from said other components in said first liquid medium prior to contacting said microparticles with a second liquid medium containing a detectable label that binds specifically to said one analyte.

Patentansprüche

1. Verfahren zum individuellen Detektieren einer Mehrzahl von Analyten in einer einzelnen biologischen Fluidprobe durch Analysen, die das Binden von Spezies in der einzelnen biologischen Fluidprobe an eine feste Phase, die in Kontakt mit einem flüssigen Medium steht, in dem die feste Phase nicht lösbar ist, und die Trennung der festen Phase von dem flüssigen Medium umfassen, wobei das Verfahren umfasst:

   Verwenden einer Mehrzahl von Mikropartikeln aus magnetisch reaktionsbereitem Material als die feste Phase, an die jeweils ein Analysereagenz gekoppelt ist, das selektiv in einer Analyse für einen der Mehrzahl von Analyten aktiv ist, wobei die Mikropartikeln hinsichtlich ihrer Größe über einen Bereich variieren, der ein Aggregat einer Mehrzahl von Unterbereichen ist, und jeder Unterbereich von anderen Unterbereichen des Aggregats durch Flusszytometrie und durch das Analysereagenz unterscheidbar ist, das an die Mikropartikeln des Unterbereichs gekoppelt ist;

   magnetisches Trennen der Mikropartikeln in allen der Unterbereiche von dem flüssigen Medium in einem einzigen Schritt; und

   Definieren des flüssigen Mediums als ein erstes flüssiges Medium, Suspendieren der von diesem getrennten Mikropartikeln in einem zweiten flüssigen Medium, Analysieren der Mikropartikeln in dem zweiten flüssigen Medium in einem einzigen Schritt durch Flusszytometrie in Übereinstimmung mit der Mehrzahl von Analysen, wodurch individuelle Detektierung der Mehrzahl von Analyten in der einzelnen biologischen Fluidprobe erzielt wird.

2. Verfahren nach Anspruch 1, bei dem eines der an die feste Phase gekoppelten Analysereagenzien ein Bindungsprotein ist, das spezifisch für einen der Analyten ist, wobei das Verfahren umfasst, zu dem ersten flüssigen Medium eine detektierbare Markierung hinzuzufügen, die sich an die feste Phase bindet, und die magnetische Trennung umfasst, die detektierbare Markierung, die an die feste Phase gebunden ist, von einer nicht gebundenen detektierbaren Markierung zu trennen, die in dem ersten flüssigen Medium suspendiert ist.

3. Verfahren nach Anspruch 2, bei dem die detektierbare Markierung dem ersten flüssigen Medium als ein Konjugat mit einer zusätzlichen Menge des einen Analyten hinzugefügt wird, wodurch das Konjugat und der eine Analyt in der Probe veranlasst werden, in Konkurrenz für das Bindungsprotein in einer Konkurrenzanalyse zu treten.

4. Verfahren nach Anspruch 2, bei dem die detektierbare Markierung dem ersten flüssigen Medium als ein Konjugat mit einer zusätzlichen Menge des einen Analyten nach einer ersten Inkubationszeitspanne hinzugefügt wird, wodurch das Konjugat in einer Sequenzanalyse veranlasst wird, mit denjenigen Stellen des Bindungsproteins, die nicht durch den einen Analyten in der Probe besetzt sind, zu reagieren.

5. Verfahren nach Anspruch 2, bei dem das Bindungsprotein als ein erstes Bindungsprotein definiert ist, und die detektierbare Markierung dem ersten flüssigen Medium als ein Konjugat mit einem zweiten Bindungsprotein hinzugefügt wird, das auch spezifisch für den einen Analyten ist, was den einen Analyten veranlasst, sich in einer Sandwichanalyse sowohl an das erste Bindungsprotein als auch das Konjugat zu binden.

6. Verfahren nach Anspruch 5, bei dem der eine Analyt ein Antigen ist, das erste Bindungsprotein ein erster Antikörper zu dem Antigen ist, und das zweite Bindungsprotein ein zweiter Antikörper zu dem Antigen ist.

7. Verfahren nach Anspruch 5, bei dem der eine Analyt ein erster Antikörper ist, das erste Bindungsprotein ein Antigen ist, und das zweite Bindungsprotein ein zweiter Antikörper mit Bindungsaffinität für den ersten Antikörper ist.

8. Verfahren nach Anspruch 1, bei dem eines der Analysereagenzien ein Bindungsprotein ist, das an die feste Phase gebunden und spezifisch für einen der Analyten und für andere Komponenten in dem ersten flüssigen Medium ist, und die magnetische Trennung umfasst, die Mikropartikeln mit dem einen daran gebundenen Analyten von den anderen Komponenten in dem ersten flüssigen Medium zu trennen, bevor die Mikropartikeln mit einem zweiten flüssigen Medium in Kontakt gebracht werden, das eine detektierbare Markierung enthält, welche sich spezifisch an den einen Analyten bindet.

Revendications

1. Procédé de détection individuelle d'une pluralité d'analytes dans un échantillon unique de fluide biologique par des méthodes de dosage qui comportent la liaison d'espèces dans ledit échantillon unique de fluide biologique à une phase solide qui est en contact avec un milieu liquide dans lequel ladite phase solide est insoluble, et la séparation de ladite phase solide d'avec ledit milieu liquide, ledit procédé comprenant :

   l'utilisation en tant que dite phase solide, d'une pluralité de microparticules de matériau magnétiquement sensible avec chacune un réactif de dosage couplé à elles qui soit sélectivement actif lors d'un dosage vis-à-vis de l'un de ladite pluralité d'analytes, lesdites microparticules variant en taille dans une plage qui soit un agrégat d'une pluralité de sous-plages, chaque sous-plage étant distinguable des autres sous-plages dudit agrégat par cytométrie en flux et par le réactif de dosage couplé aux microparticules de ladite sous-plage ;
   la séparation par magnétisme, dans l'ensemble des sous-plages, des microparticules d'avec ledit milieu liquide en une seule étape, et
   la définition dudit milieu liquide comme un premier milieu liquide, la suspension desdites microparticules qui en sont séparées dans un second milieu liquide, l'analyse desdites microparticules dans ledit second milieu liquide, en une seule étape, par cytométrie en flux en accord avec ladite pluralité de dosages, menant ainsi à bien la détection individuelle de ladite pluralité d'analytes dans ledit échantillon unique de fluide biologique.

2. Procédé selon la revendication 1, dans lequel un desdits réactifs de dosage couplés à ladite phase solide est une protéine liante spécifique de l'un desdits analytes, ledit procédé comprend l'ajout audit premier milieu liquide d'un marqueur détectable qui se lie à ladite phase solide, et ladite séparation magnétique comprend la séparation du marqueur détectable qui est lié à ladite phase solide d'avec un marqueur détectable non lié qui est suspendu dans ledit premier milieu liquide.

3. Procédé selon la revendication 2, dans lequel ledit marqueur détectable est ajouté audit premier milieu liquide sous forme d'un conjugat avec une quantité supplémentaire dudit un analyte, provoquant une compétition dudit conjugat et dudit analyte dans ledit échantillon pour ladite protéine liante dans un dosage de type compétitif.

4. Procédé selon la revendication 2, dans lequel ledit marqueur détectable est ajouté audit premier milieu liquide sous forme d'un conjugat avec une quantité supplémentaire dudit un analyte après une première période d'incubation, provoquant la réaction dudit conjugat avec ceux des sites de ladite protéine liante qui ne sont pas occupés par ledit un analyte dans ledit échantillon dans un dosage séquentiel.

5. Procédé selon la revendication 2, dans lequel ladite protéine liante est définie comme une première protéine liante, et ledit marqueur détectable est ajouté audit premier milieu liquide sous forme d'un conjugat avec une seconde protéine liante qui est également spécifique dudit un analyte, provoquant la liaison dudit un analyte à la fois à ladite première protéine liante et audit conjugat dans un dosage à double site en phase solide (ou de type sandwich).

6. Procédé selon la revendication 5, dans lequel ledit un analyte est un antigène, ladite première protéine liante est un premier anticorps dudit antigène, et ladite seconde protéine liante est un second anticorps dudit antigène.

7. Procédé selon la revendication 5, dans lequel ledit un analyte est un premier anticorps, ladite première protéine liante est un antigène, et ladite seconde protéine liante est un second anticorps ayant une affinité de liaison pour ledit premier anticorps.

8. Procédé selon la revendication 1, dans lequel un desdits réactifs de dosage est une protéine liante liée à ladite phase solide et spécifique de l'un desdits analytes et d'autres composants dans ledit premier milieu liquide, et ladite séparation magnétique comprend la séparation desdites microparticules avec ledit un analyte lié à elles d'avec lesdits autres composants dans ledit premier milieu liquide avant de mettre en contact lesdites microparticules avec un second milieu liquide contenant un marqueur détectable qui se lie spécifiquement audit un analyte.